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Analysis of Genetic Diversity in the VP1 Unique Region Gene of Human Parvovirus B19 Using the Mismatch Detection Method and Direct Nucleotide Sequencing

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To assess the prevalent genetic types of human parvovirus B19 strains derived from various sources and their relation to particular clinical symptoms, the genetic diversity in the VP1 unique region, which is important for the neutralizing response to human parvovirus B19, was examined by the mismatch detection method using the Non-isotopic RNase Cleavage Assay™ (NIRCA™) and direct nucleotide sequencing. Twenty three samples obtained between 1986 and 1997 were examined. Three electrophoresis patterns were observed with NIRCA™. The nucleotide sequence showed that there were 14 nucleotide changes and 4 amino acid substitions in comparison with Au strains employed as a standard strain. The nucleotide variability of all samples ranged from 0.3 to 2.7% and the amino acid variability ranged from 1.0 to 3.0%. They were classified into three types according to NIRCA™. Types 1 and 3 had similar sequences, but the type 2 sequence was quite different. Although there were some nucleotide variations in the same NIRCA™ type, these were silent. However, there was no relationship between the clinical features and NIRCA™ types or between clinical features and the nucleotide sequence. All samples obtained before 1987 were NIRCA™ type 2. On the other hand, 19 of 20 samples obtained after 1989 were NIRCA™ type 1. The other sample obtained in 1992 was type 3. The results suggest that the B19 strain of type 2 disappeared by 1988 and changed to other B19 strains such as type 1 and type 3 after 1988, indicating a correlation between genome type and prevalence. NIRCA™ is a convenient method for screening mutations due to its simplicity and quickness. ***J. Med. Virol. 56:205–209, 1998.***
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KEY WORDS: human parvovirus B19; VP1 unique region; mismatch detection method; non-isotopic RNase cleavage assay

INTRODUCTION

Human parvovirus B19 (B19) causes erythema infectiosum [Anderson et al., 1983], hydrops fetalis [Brown et al., 1984], and aplastic crisis in patients with hematologic disorders [Pattison et al., 1981]. B19 has tissue tropism for erythroid precursor cells and the receptor of the virus is globoside (blood group P antigen) [Brown et al., 1993]. Globoside exists on the surface of erythroid cells, hepatocytes, myocardial cells, and vascular endothelial cells [von dem Borne et al., 1986]. The cellular distribution of the receptor is responsible for B19 causing hepatitis, myocarditis, and vasculitis.

B19 has two viral capsid proteins, VP1 and VP2. They have the same open reading frame and VP1 is identical to VP2 except for an additional 227 amino acids at the NH_2-terminus. Therefore, these additional amino acids are referred to as the VP1 unique region [Shade et al., 1986; Ozawa et al., 1987]. Over 95% of capsid proteins are VP2, while VP1 accounts for less than 5%. Although virion consists of less than 5% of VP1, this unique region contains multiple linear neutralizing epitopes [Saikawa et al., 1993]. Recombinant empty capsids containing VP2 cannot elicit a strong neutralizing antibody response. Therefore, VP1 is thought to be important in the immune response for neutralizing B19 [Rosenfeld et al., 1992; Bansal et al., 1993; Kawase et al., 1995]. In this study, genetic diversity was examined in the VP1 unique region of B19 by the mismatch detection method and by direct nucleotide sequencing. The prevalent genetic types of human parvovirus B19 strains and their relation to particular clinical symptoms were also studied.

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TABLE I. List of Analyzed Samples

Sample no.	Age (Year)	Sex	Sample	Year
1	13	F	Serum	1986
2	Unknown	F	Serum	1987
3	4	M	Serum	1987
4	18	M	Serum	1989
5	11	F	Serum	1990
6	4	M	Cerebrospinal fluid	1990
7	4	M	Cerebrospinal fluid	1990
8	5	F	Serum	1990
9	15	M	Serum	1990
10	9	M	Serum	1991
11	11	F	Serum	1991
12	5	M	Cerebrospinal fluid	1991
13	Unknown	F	Serum	1992
14	Unknown	F	Serum	1992
15	Unknown	F	Serum	1992
16	8	M	Serum	1993
17	8	F	Serum	1994
18	Unknown	M	Serum	1994
19	Unknown	F	Serum	1995
20	4	M	Serum	1995
21	9	M	Serum	1995
22	4	F	Serum	1996
23	12	F	Serum	1997

MATERIALS AND METHODS

Twenty samples of sera and three of cerebrospinal fluid obtained between 1986 and 1997 (Table I) were studied. These samples were B19 DNA positive by nested polymerase chain reaction (PCR) and were stored at −70°C until tested in this study. Nested PCR was carried out by a method described previously [Yoto et al., 1993]. Briefly, the first PCR was undertaken with the primers of 3503–3522 and 3882–3901. The second PCR was undertaken with the primers 3542–3561 and 3847–3867 in the VP region. A 10 μl sample was incubated at 70°C for 1 minute to inactivate the inhibitory factor in the sample and was used directly as a template, according to the method of Frickhofen and Young [1991]. Samples were retested.

Genetic diversity in the VP1 unique region of B19 was analyzed by the mismatch detection method using the Non-isotopic RNase Cleavage Assay™ kit (NIRCA™, Ambion Inc., Austin, TX) and direct nucleotide sequencing. NIRCA™ was adapted from a screening method of point mutation first described by Myers et al. [1985] and Winter et al. [1985]. This method is based on the observation that RNase A is able frequently to cleave a single unpaired base. Although the substrates for RNase A digestion were RNA/DNA duplexes in the original reports, the target substrates for RNase digestion are RNA/RNA duplexes in NIRCA™. This assay was carried out according to the instruction manual and a B19 clone (a gift from Dr. Clewley) was used as a control.

Nested PCR Assay for NIRCA™

After incubation at 70°C for 1 minute, 10 μl of the specimen or control was added to the first round of the PCR mixture (90 μl) containing a reaction buffer (final concentration: 10 mmol/l Tris-HCl; 50 mmol/l KCl; 1.5 mmol/l $MgCl_2$; 200 μmol/l each of dATP, dCTP, dGTP, dTTP; 2.5 U of Taq polymerase; and 100 nmol/l each of the primers 2300–2319 and 2795–2814). After the first round of PCR amplification, 2 μl of the first PCR product was transferred into a second-round PCR mixture (100 μl) which contained the same constituents as the initial mixture except for the primers. The primers for the control were 2408–2427 and 2741–2760, which has a T7 phage promoter sequence (5′-TAATACGACTCACTATAGGG-3′) in the 5′ terminal. The primers for the control and the test specimens were 2408–2427, which has an SP6 phage promoter sequence (5′-ATTTAGGTGACACTATA-3′) in the 5′ terminal, and 2741–2760. Thirty cycles of both first and second-round amplifications were carried out at 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 2 minutes. We used a serum sample from a healthy volunteer who had previously been confirmed to be not infected with B19 as a negative control of nested PCR. Samples were retested to verify that positive results were not false-positive.

NIRCA™

Two microliters of the final PCR product, including the negative control of nested PCR, was added to the transcription mixture (8 μl) containing a reaction buffer (2 μl of 2.5 mmol/l rNTP mixture; 1 μl of SP6 or T7 RNA polymerase [20 U/μl]; 1 μl of 10× transcription buffer, 4 μl of deionized water). The mixture was incubated at 37°C for 1 hour (in vitro transcription). The transcript was generated using T7 RNA polymerase in the control and SP6 RNA polymerase in the control and test samples. An equal volume of mismatch hybridization buffer was added to the mixture and incubated at 95°C for 3 minutes. Ten microliters of the transcript from the control and 10 μl of that from the control or test samples were mixed and incubated at 95°C for 3 minutes to hybridize. Four microliters of the hybridized product (RNA/RNA duplex) was treated with 16 μl of diluted RNase A solution at 37°C for 45 minutes. The RNase cleavage product with 4.5 μl of mismatch gel loading solution was electrophoresed on 2% agarose gel in 1× Tris-borate/EDTA electrophoresis buffer (Fig. 1).

Nested PCR and NIRCA™ were done in batches. Nucleotide sequences of PCR products were determined by an automated sequencer (ABI 373S, Applied Biosystems Division PERKIN ELMER, CA).

RESULTS

Three types of RNase cleavage products were obtained by NIRCA™ and referred to as types 1–3 according to the number of cleavage fragments (Fig. 2). RNase cleavage product obtained from a negative control had no band (data not shown) and RNase cleavage product using RNA/RNA duplex generated from the B19 clone only had one band (original band). The samples were examined three times to validate the re-

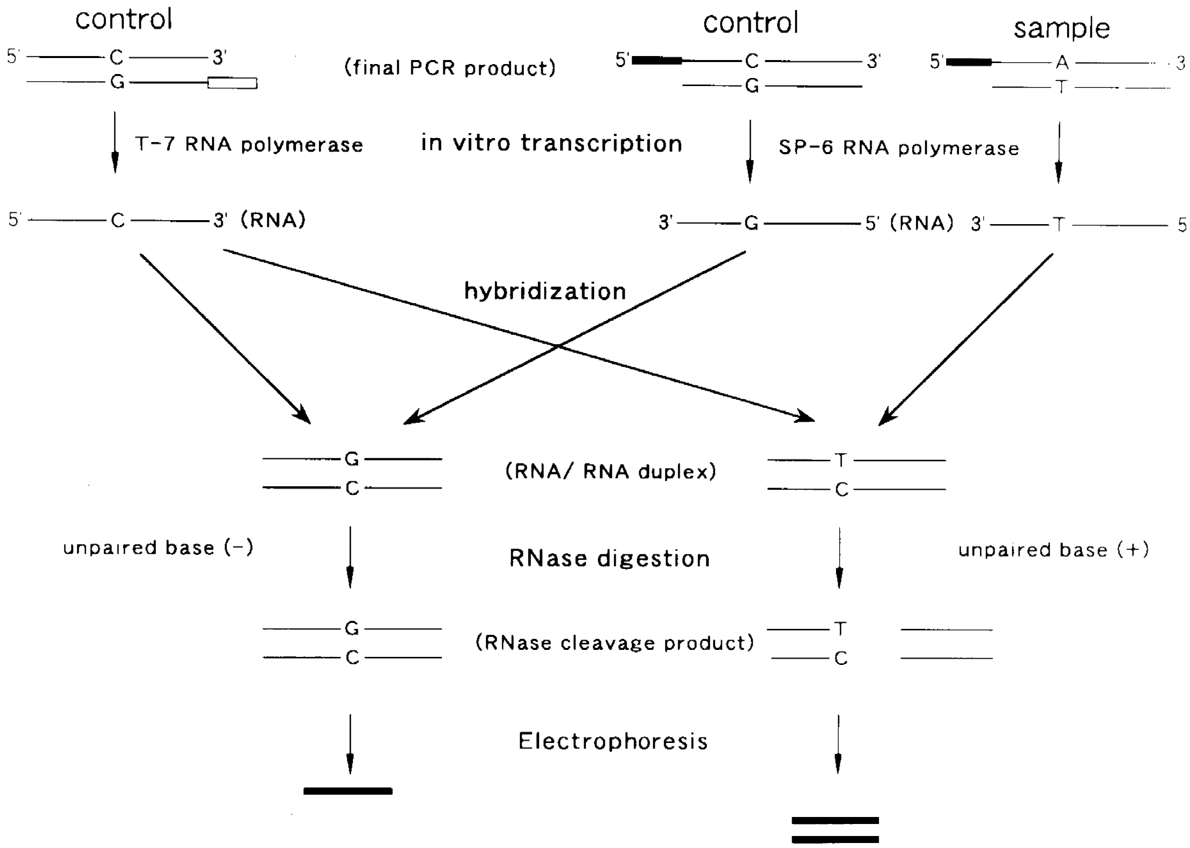

Fig. 1. Scheme of NIRCA™. RNA was transcribed using T7 RNA polymerase in the control and SP6 RNA polymerase in the control and test samples. The transcript from the control and that from the control or the test samples were hybridized. RNA/RNA duplex was treated with RNase A. The RNase cleavage product was electrophoresed. The open box means a T7 phage promoter sequence and the closed box means an SP6 phage promoter sequence.

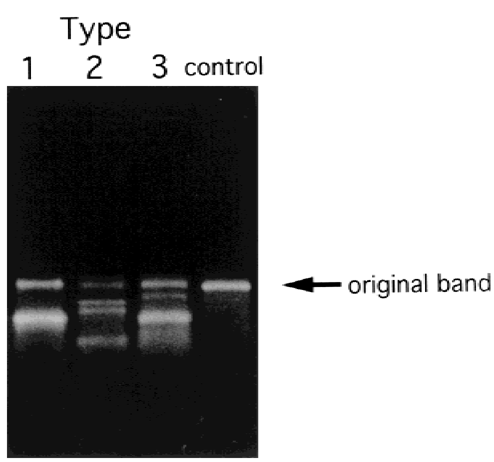

Fig. 2. NIRCA™ analysis of RNA/RNA duplicate. RNA/RNA duplex was generated from the PCR product of a sample and that of B19 cloned DNA. Control RNA/RNA duplex was generated from the PCR product of a B19 clone only. They were incubated with RNase at 37°C for 45 minutes and cleavage product was electrophoresed with loading buffer containing ethidium bromide on 2% agarose gel. Control RNA/RNA duplex had no mismatch. Three different electrophoresis patterns were observed (types 1, 2 , and 3).

producibility. Type 1 consists of only one fragment in addition to the original band, while types 2 and 3 consist of three and two fragments, respectively. Among the 23 samples tested, 19 were type 1, 3 were type 2, and 1 was type 3. Thus, type 1 accounted for 82.6% of the samples. All samples had their own and common RNase cutting sites and were different from the control clone.

We analyzed the nucleotide sequences of each type of PCR product and compared the nucleotide sequence of our isolates with the Au strain whose sequence has been published [Shade et al., 1986] (Table II). In the VP1 unique region analyzed, there were 14 nucleotide changes in comparison with the Au strain. The variability of all samples ranged from 0.3 to 2.7%. All samples had one common nucleotide substitution at nucleotide 2453, which led to one amino acid change in comparison with the Au strain. Type 1 was classified into five different sequences (A to E) and type 2 into three different sequences (F to H) according to the position and number of nucleotide substitutions. Type 1 had at least five additional nucleotide substitutions, which were silent for the amino acid replacement. Type 2 had an additional seven substitutions, which led to two amino acid changes. This corresponds to the three

TABLE II. Comparison of Nucleotide Sequence and Amino Acid Between Au Strain and Samples

		NIRCA™	Type 1 (n = 19)					Type 2 (n = 3)			Type 3 (n = 1)	
Nucleotide	Au	Sequence	A (10)	B (6)	C (1)	D (1)	E (1)	F (1)	G (1)	H (1)	I	Amino acid change
2453	A		G	G	G	G	G	G	G	G	G	Lys→Glu
2490	C										T	Ala→Val
2500	G								C	C		—
2504	C							G	G	G		Gln→Glu
2515	G							A	A	A		—
2527	A							G	G	G		—
2530	G				A							—
2539	A			T							T	—
2545	C							T	T	T		—
2554	T							C	C			—
2594	A							C	C	C		Asn→His
2618	C						T					—
2659	C						T					—
2689	A					G						—

cleavage fragments obtained by NIRCA™, which suggests mismatch frequency. Type 3 had one more additional nucleotide substitution in sequence B of type 1, which led to one amino acid change.

Four amino acid changes were found in the region analyzed. The variability of all samples ranged from 1.0 to 3.0%. Among the four amino acid changes, one was a substitution from a neutral amino acid (asparagine) to a basic amino acid (histidine), one was from a neutral amino acid (glutamine) to an acidic amino acid (glutamic acid), and one was from a basic amino acid (lysine) to an acidic amino acid (glutamic acid). Although type 1 consisted of five different sequences and type 2 consisted of three, none of them resulted in amino acid changes. Although there was one common amino acid change, other amino acid changes were restricted in certain type under the classification of this NIRCA™ assay. The clinical features and nucleotide sequence were compared to find a possible association (Table III). However, no specific correlation was found between clinical features and nucleotide sequence even in two samples associated with a unique symptom of encephalopathy.

TABLE III. Comparison Among Clinical Manifestation, NIRCA™ Type, and Sequence

Clinical manifestations[a]	NIRCA™ type	Sequence	Sample no.	Year
Anemia	1	A	4	1989
Aplastic crisis, HS	1	A	5	1990
Encephalopathy, EI	1	A	6	1990
Encephalopathy	1	A	7	1990
EI	1	A	8	1990
Encephalopathy, EI	1	A	9	1990
Anemia	1	A	10	1991
Anemia	1	A	11	1991
Encephalopathy	1	A	12	1991
EI	1	A	13	1992
Pancytopenia	1	B	14	1992
EI	1	B	19	1995
Asymptomatic	1	B	20	1995
EI, liver dysfunction	1	B	21	1995
Aplastic crisis, HS	1	B	22	1995
EI	1	B	23	1997
EI	1	C	16	1993
Asymptomatic	1	D	17	1994
EI	1	E	18	1994
Aplastic crisis, IDA	2	F	1	1986
Aplastic crisis, HS	2	G	2	1987
EI	2	H	3	1987
Aplastic crisis, HS	3	I	15	1992

[a]EI, erythema infectiosum; HS, hereditary spherocytosis; IDA, iron deficiency anemia.

DISCUSSION

B19 has two viral capsid proteins, VP1 and VP2. In this study, the genetic diversity of the VP1 unique region, which is thought to be important in the immune response for neutralizing B19, was investigated. On NIRCA™, using the mismatch detection method, three electrophoresis patterns were observed by RNase digestion of RNA/RNA duplicates from samples and a control clone. Direct sequencing of PCR products in this region revealed that there was one common nucleotide change (nt 2453) among all types and some nucleotide differences in the same NIRCA™ type. However, these nucleotide substitions were all silent, and it was thought that proteins coded by this region have the same antigenicity.

Three samples obtained before 1987 were all NIRCA type 2. In the remaining 20 samples, which were obtained after 1989, 19 were NIRCA type 1 and only one was NIRCA type 3. The sample had the same nucleotides of sequence B except for one nucleotide (nt 2490). This nucleotide change resulted in an amino acid change. Types 1 and 3 were closely related, but type 2 had a different sequence from type 1 and type 3. From the point of view of molecular epidemiology, type 2 was not prevalent after 1987 and types 1 and 3 were prevalent after 1989. These data indicate that certain genome types predominate in each epidemic. Erdman et al. [1996] reported that multiple B19 lineages can cocirculate, although one B19 genotype may predominate in a single communitywide outbreak, and that genotypes may have geographical specificity.

Mori et al. [1987] classified four types of B19 strain using 13 restriction enzymes. Genetic variability of viruses has been discussed in correlation with particular

clinical manifestations of infection. However, no correlation has been demonstrated between genetic variability and clinical manifestations. Hemauer et al. [1996] reported that isolates from patients in whom infection persisted exhibited a high degree of variability in the NS-1 and VP coding regions. This was especially so in the VP1 unique region in which unpolar, uncharged residues were often replaced by polar or charged amino acids. Regarding the VP1 unique region analyzed in the present study, there were no samples from persistent infection. Nor were changes from unpolar, uncharged residues to polar or charged amino acids. Although B19 strain variation has been demonstrated even in a narrow portion of the VP1 unique region, no correlation was found among nucleotide sequence, amino acid change, and specific clinical manifestation.

Another simple method of PCR-based techniques for mutation detection is the single-strand conformation polymorphism (SSCP) assay. Kerr et al. [1995] detected mutations occurring in the nonstructural region of B19 DNA using PCR-SSCP, but all these were silent and no correlation was observed between nucleotide changes and clinical illness or the patient's age. They suggested that study of the sequence encoding the VP1 unique region might be appropriate for interpretation of B19 epidemiology and clinical correlations. However, a genetic association with common clinical presentations was not apparent in our study of this region. Further investigation should be undertaken to resolve these issues. The mismatch detection method is also a very useful technique for screening mutations from a large number of B19 isolates due to its sensitivity, simplicity, and reproducibility.

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